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Inhibitory and Stimulatory Effects of *Pseudomonas aeruginosa* Pyocyanine on Human T and B Lymphocytes and Human Monocytes

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Pyocyanine, a pigment produced by *Pseudomonas aeruginosa*, has dual dose-dependent stimulatory as well as inhibitory effects on immune responses in vitro as measured by DNA synthesis of human T and B lymphocytes, interleukin-2 (IL-2) production by human T lymphocytes, immunoglobulin production by human B lymphocytes, and monokine production by human monocytes. In general, stimulatory activity was found at low concentrations of pyocyanine, whereas high concentrations of the pigment resulted in an inhibition of responses. At a pyocyanine concentration of 0.1 μg/ml or less the proliferation of T and B lymphocytes was enhanced, but at 0.5 μg/ml it was suppressed. IL-2 production by T lymphocytes was enhanced at concentrations up to 0.5 μg/ml but totally inhibited at 1.0 μg/ml. The differentiation of B lymphocytes to become immunoglobulin-producing cells was also enhanced in the presence of low doses of pyocyanine, whereas secretion of immunoglobulin by B lymphocytes was suppressed at all concentrations of pyocyanine. In contrast to the dual effects of pyocyanine on lymphocyte response, lipopolysaccharide-induced IL-1 and tumor necrosis factor release by monocytes was markedly enhanced by low as well as high concentrations of pyocyanine. From these results we conclude that this property of pyocyanine may lead to suppression of specific defense mechanisms and enhance harmful inflammatory reactions of the host during infection with *Pseudomonas aeruginosa*.

Infection with *Pseudomonas aeruginosa* is an important clinical problem in compromised patients, e.g., those with burns, cystic fibrosis, or neoplastic disease, and often results in severe morbidity or even mortality. The gram-negative bacteria and their various products have been shown to affect the specific and nonspecific defense mechanisms of the host: (i) heat-killed *P. aeruginosa* or a lyophilizate of this bacteria inhibits immune responses in vivo (6, 23) as well as in vitro (12, 27); (ii) alkaline proteases and elastase are exoproteins of the bacteria which inhibit T-lymphocyte and natural killer cell functions in vitro (22, 31) and which may contribute to the virulence of these bacteria in pulmonary infections (2, 33); and (iii) exotoxin A is another extracellular product of *P. aeruginosa* with immunomodulating properties that is considered to play a significant role in pathogenicity (2, 10).

This paper deals with pyocyanine, a bacterial product which is released by most clinical *P. aeruginosa* isolates (14, 15). It was first extracted in 1860 from wound dressings and crystallized with chloroform as described by Fordos (7). Pyocyanine (*N*-methyl-1-hydroxyphenazine) is a blue phenazine pigment with a molecular weight of 210.23 (Fig. 1). It has been reported that pyocyanine inhibits the T-lymphocyte response to antigens and mitogens in vitro (20, 21, 30) and superoxide production by neutrophils (17). In all reports published so far, inhibition of the T-lymphocyte response was accompanied by a reduction of interleukin-2 receptor (IL-2R) expression by the cells (20, 21). With respect to the production of IL-2, the results published so far are contradictory. Whereas Mühlradt et al. (20) did not find an inhibition of IL-2 production in murine T cells by pyocyanine, Nutman et al. (21) reported an inhibition of IL-2 production after mitogenic stimulation of human T lymphocytes.

In the present study, the influence of pyocyanine on mitogen-induced production of immunoglobulins and the polypeptide hormones (cytokines) IL-1, tumor necrosis factor (TNF), and IL-2 was investigated. We found that pyocyanine has both stimulatory and inhibitory effects on cellular responses. In this way pyocyanine may be involved in the inhibition of specific immune responses but also enhances nonspecific inflammatory reactions, which have fatal consequences in infected patients.

MATERIALS AND METHODS

Preparation of synthetic pyocyanine. Synthetic pyocyanine was prepared from phenazine methosulfate by a photochemical reaction as described previously (14). Phenazine methosulfate was dissolved in Tris hydrochloride buffer (0.01 mol/liter [pH 7.23]). The photochemical reaction was performed by illumination for 2 days at room temperature. The blue products were extracted with chloroform, evaporated, and dissolved again in methanol. The methanol solution was adsorbed to silica gel, and pyocyanine was purified by thin-layer chromatography with methanol-chloroform (1:1) as the solvent system. The blue spot with an R_f of 0.65 was extracted with chloroform, the silica gel was discarded after centrifugation, and the solution was evaporated and dissolved in methanol. After drying under nitrogen, the remaining material was dissolved in 0.85% NaCl and incubated overnight at 4°C. The resulting blue crystals were isolated by centrifugation. The absorption spectrum of this synthetic pyocyanine is the same as described previously (34).

Isolation of cells. Peripheral blood mononuclear cells (MNC) were isolated from heparinized blood from healthy

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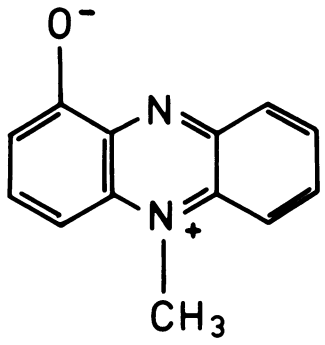

FIG. 1. Chemical structure of pyocyanine. Modified from reference 18.

donors by density gradient centrifugation on Ficoll-Paque as described earlier (32). After density gradient centrifugation the cells were washed three times (250 × *g* for 10 min each) and suspended in the desired culture medium.

Cell cultures. Stimulation of DNA synthesis, IL-2 receptor expression, and IL-2 production by T lymphocytes were performed as described elsewhere (28). In brief, MNC were suspended at 2×10^5 cells per ml in culture medium (RPMI 1640 supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml) plus 10% fetal calf serum (FCS; Biochrom, Berlin, Federal Republic of Germany). The cells were stimulated with 1 μg of phytohemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, England) per ml in the presence or absence of pyocyanine, and 200-μl portions were cultured in flat-bottom microdilution plates (Greiner, Nürtingen, Federal Republic of Germany). DNA synthesis and IL-2 receptor expression usually were determined after a culture period of 4 days. Cultures for stimulation of IL-2 production by T lymphocytes were performed at 5×10^5 MNC per ml in culture medium supplemented with 2% FCS. The culture supernatants were harvested after 2 days of culture or, in kinetics experiments, at different times after the start of culture.

B lymphocytes were stimulated as described elsewhere (24, 25). In brief, MNC (1×10^6/ml or 5×10^5/ml) were suspended in culture medium plus 10% FCS, and 200-μl portions were cultured in flat-bottom microdilution plates (Greiner). A 10% (wt/vol) suspension of *Staphylococcus aureus* Cowan 1 (SAC; Calbiochem, Frankfurt am Main, Federal Republic of Germany) at 10 μg/ml or pokeweed mitogen (PWM; Biochrom) at 0.4 μg/ml was used to stimulate the cells. DNA synthesis in B lymphocytes was determined after 3 days of culture. The number of immunoglobulin-secreting cells (ISC) and the number of immunoglobulin-producing cells (IPC; cells with intracytoplasmatic immunoglobulin) were measured after 6 days of culture.

IL-1 and TNF production by monocytes was stimulated as described elsewhere (5a). In brief, MNC were suspended in culture medium (without FCS) at 4×10^6 cells per ml, and 200-μl portions were cultured in round-bottom microdilution plates (Greiner) in the presence or absence of pyocyanine. For activation of IL-1 and TNF production, the cells were stimulated with *S. abortusequii* lipopolysaccharide (LPS; kindly provided by H. Brade, Forschungsinstitut Borstel). After a 24-h culture, the culture supernatants were harvested and tested for IL-1 and TNF activity.

Determination of DNA synthesis. For the determination of DNA synthesis, the cells were pulsed for 5 h (T lymphocytes) or 16 h (B lymphocytes) with [^{3}H]thymidine (0.2 μCi per culture; specific activity, 2 Ci/mmol; The Radiochemical Centre, Amersham, England). The cells were collected on fiberglass filters by using a 96-well plate harvester (Biotec, Gelterkirchen, Switzerland), and the filters were processed for measurement in a liquid scintillation counter.

Determination of IL-2 activity. The supernatants of stimulated cultures were diluted 1:3 in a series of eight steps. The growth-promoting activity of IL-2 was determined by the addition of an IL-2-dependent murine T-cell line (CTL-6) as described previously (3). After 1 day of culture the cells were pulsed with 0.2 μCi of [^{3}H]thymidine per culture for 5 h and processed for liquid scintillation counting as described above. The IL-2 activity was calculated by probit analysis (8) by using a laboratory standard containing 68 international reference units per ml. Pyocyanine carried over in the culture supernatants into the IL-2 assay did not affect the outcome of the determination.

Determination of CD25-bearing cells. CD25-positive cells were determined by using the anti-Tac monoclonal antibody (kindly provided by T. A. Waldmann, National Institutes of Health, Bethesda, Md.). Indirect immunofluorescence of anti-Tac binding was achieved by using affinity-purified fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin G (IgG) (Dianova GmbH, Hamburg, Federal Republic of Germany) as a second antibody. Immunofluorescence-labeled MNC were fixed in phosphate-buffered saline containing 1.5% paraformaldehyde and than applied to a cytofluoragraf (System 50; Ortho Diagnostics, Inc., Raritan, N.J.) for fluorescence analysis. Only lymphocytes, but not monocytes, were counted by light scatter-gated exclusion.

Determination of IL-1 activity. IL-1 in the culture supernatants was determined by using human foreskin fibroblasts as described previously (16). The supernatants from endotoxin-stimulated MNC were diluted 1:4 in a series of six steps. The growth-promoting activity of IL-1 on fibroblasts was determined by addition of the supernatants to fibroblast cultures in flat-bottom microdilution plates. After cultivation for 4 days, the supernatants were discarded and the cells were washed with tap water and stained with crystal violet. After the dye had been dissolved in 0.5% sodium dodecyl sulfate, the optical density at 550 nm was determined in a Microelisa Auto Reader (Dynatech Deutschland, Denkendorf, Federal Republic of Germany). IL-1 activity was calculated by probit analysis by using a laboratory standard with 1,000 international reference units per ml. Pyocyanine carried over in the culture supernatants into the IL-1 assay did not affect the outcome of the results.

Determination of TNF activity. TNF in the culture supernatants was determined by using murine fibrosarcoma L929 cells as target cells for the cytotoxic activity of the monokine as described earlier (5a). The culture supernatants of LPS-stimulated MNC were diluted in seven steps of 1:3 in flat-bottom microdilution plates (Becton Dickinson, Heidelberg, Federal Republic of Germany), and dactinomycin (1 μg/ml) and L929 cells (5×10^4 cells per culture) were added. After incubation for 19 h the supernatants were discarded, and the remaining (live) cells were stained with crystal violet and further processed as described above. The TNF concentrations in the samples were calculated by probit analysis with a standard of recombinant TNF (kindly provided by BASF-Knoll AG, Ludwigshafen, Federal Republic of Germany) with 40 international reference units per ng of protein. Pyocyanine carried over in the culture supernatants into the TNF assay did not affect the outcome of the results.

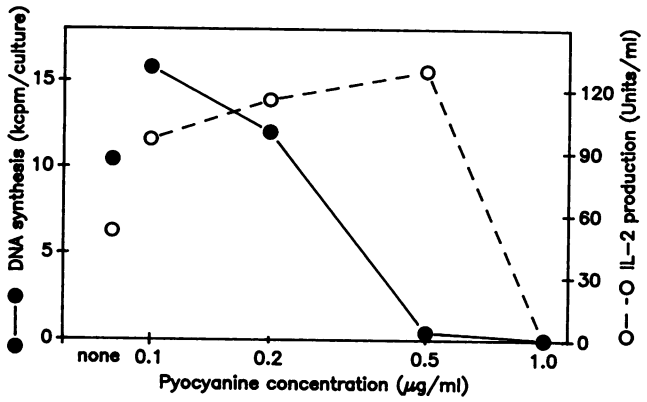

FIG. 2. Pyocyanine dose-response curves for DNA synthesis and IL-2 production of PHA-stimulated T lymphocytes. Human MNC were stimulated with PHA (1 μg/ml) in the absence or presence of various amounts of pyocyanine. Production of IL-2 by the cells was measured after 2 days of culture, and DNA synthesis was determined after 4 days of culture. The results are expressed as units per milliliter or as cpm per culture, respectively. Each value represents the mean of triplicate cultures. The standard deviation was less than 15%. When cells were cultured in the presence of various doses of pyocyanine in the absence of PHA, the values never exceeded the control values.

Determination of ISC and IPC. After completion of the B-lymphocyte cultures, the cells were washed three times and harvested. ISC were evaluated by the reverse plaque assay as described previously (26). IPC were determined by fluorescence microscopy after staining the fixed cells with fluorescein-labeled rabbit anti-human IgG, IgM, and IgA antibody (Dakopatts, Hamburg, Federal Republic of Germany) as described elsewhere (25).

RESULTS

Effect of pyocyanine on mitogenic stimulation of T lymphocytes. IL-2 is a peptide secreted by T lymphocytes after mitogenic stimulation. This lymphokine is involved in specific defense mechanisms of the host against foreign antigens or organisms. The results published so far on the effects of pyocyanine on Il-2 production have been contradictory, as mentioned above. In our investigation we have taken up this question again and examined the mitogenic response of human T lymphocytes in the presence of different concentrations of pyocyanine.

Human MNC were stimulated by PHA in the absence or presence of different amounts of pyocyanine. Primary experiments gave the optimal conditions for stimulation of the T lymphocytes (2×10^5 MNC per culture, 10% FCS, and 1 μg of PHA per ml). We found dose-dependent stimulatory as well as inhibitory activities of this pigment on the cells. Pyocyanine at 0.1 μg/ml enhanced DNA synthesis and IL-2 production by T cells (Fig. 2). When higher concentrations of pyocyanine were added, distinct activities of the cells were observed. Whereas the addition of 0.2 and 0.5 μg of pyocyanine per ml inhibited DNA synthesis, IL-2 release was still enhanced; however, at 1 μg of pyocyanine per ml, IL-2 release was totally abolished. These experiments were run under similar conditions four times with cells from different donors. The experiments had comparable results. Figure 2 shows the results of a typical experiment.

Kinetics studies in which pyocyanine at 0.1 or 0.5 μg/ml was added to the culture at various times indicated that the

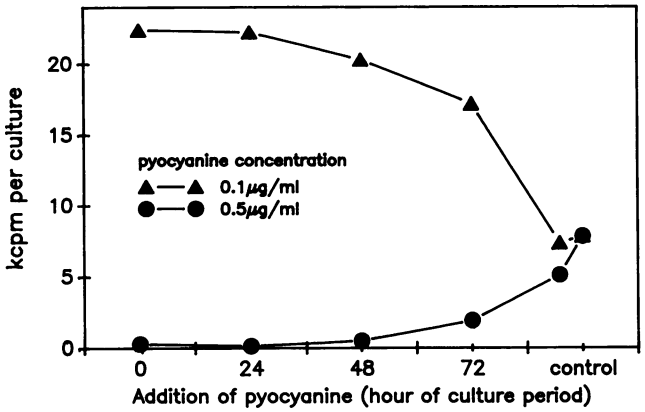

FIG. 3. Kinetics of the effect of pyocyanine on DNA synthesis by PHA-stimulated T lymphocytes. Human MNC were stimulated with PHA (1 μg/ml), and pyocyanine (0.5 or 0.1 μg/ml) was added at the indicated times. Control cultures were run in the presence of PHA but in the absence of pyocyanine. For further details, see the legend to Fig. 2.

pigment has stimulatory or inhibitory activities even when added after 72 h of culture, i.e., 24 h before the end of the cultures (Fig. 3). The time course of DNA synthesis after stimulation of MNC with PHA in the presence or absence of pyocyanine shows that the stimulatory effect of 0.1 μg of the pigment per ml can be observed up to day 5 of culture but that the inhibitory effect of 0.4 μg/ml was seen over the total period of 7 days (Fig. 4). IL-2 production was delayed by the addition of pyocyanine (Fig. 5): after 24 h of culture the supernatants of PHA-stimulated MNC contained no IL-2 (in the presence of 0.4 μg of pyocyanine per ml) or less IL-2 than the control cultures (PHA alone) (in the presence of 0.1 μg of pyocyanine per ml). However, after a prolonged culture period (48 h or more) the culture supernatants contained more IL-2 when the cells were cultured in the presence of pyocyanine.

We also tested the cytotoxicity of our pyocyanine preparations. Pyocyanine up to 0.5 μg/ml was not toxic during stimulation of human MNC with PHA for 4 days. However,

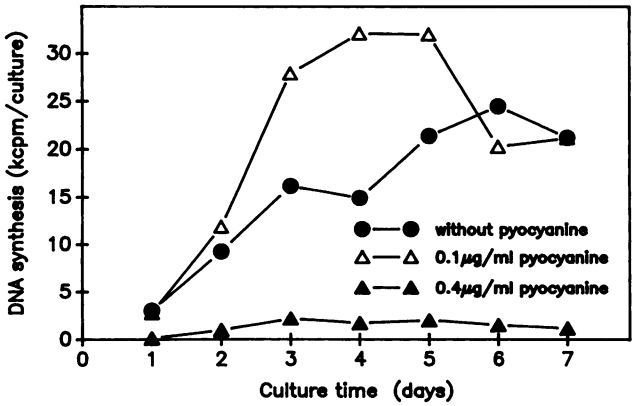

FIG. 4. Kinetics of DNA synthesis by PHA-stimulated MNC cultured in the presence or absence of pyocyanine. Human MNC were stimulated with PHA (1 μg/ml) in the absence or presence of 0.4 or 0.1 μg of pyocyanine per ml. The DNA synthesis was determined after various days of culture. For further details, see the legend to Fig. 2.

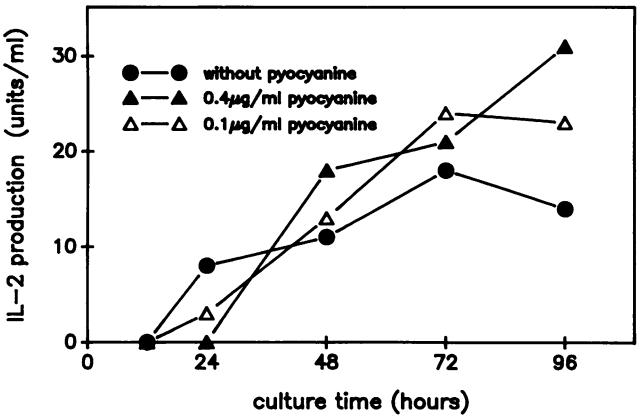

FIG. 5. Kinetics of the IL-2 release by PHA-stimulated MNC cultured in the presence or absence of pyocyanine. Human MNC were stimulated with PHA (1 μg/ml) in the absence or presence of 0.4 or 0.1 μg of pyocyanine per ml. The production of IL-2 by the cells was measured after various culture periods as indicated in the figure. For further details, see the legend to Fig. 2.

the pigment reduced the viability of the cells to about 50% when the cells were stimulated in the presence of 1.0 μg of pyocyanine per ml as determined by trypan blue exclusion (data not shown).

In a further experiment we compared the effect of pyocyanine on DNA synthesis with the expression of CD25 after mitogenic stimulation (Table 1). Again, a stimulatory effect of pyocyanine on PHA-induced DNA synthesis was noted at 0.1 μg of pyocyanine per ml, whereas the proliferative response was blocked at 0.5 μg/ml or higher. Pyocyanine reduced the expression of CD25 in a dose-dependent manner, as determined by the number and fluorescence intensity of CD25-positive cells. In contrast to the mitogenic induction of DNA synthesis and IL-2 production, the expression of CD25 receptors was not increased in the presence of 0.1 μg of pyocyanine per ml. This experiment was run twice and gave equivalent results. Table 1 shows the results of one of these experiments.

Effect of pyocyanine on mitogenic stimulation of B lymphocytes. Production of specific antibodies by B lymphocytes against foreign antigens is a vital element of the adaptive immune response. We examined the effect of pyocyanine on proliferation of and immunoglobulin production by lymphocytes. Again, previous experiments have given us the optimal conditions for B-lymphocyte stimulation. Only these optimal conditions were used throughout our experiments. DNA synthesis induced by SAC and by PWM (also a T-cell mitogen) was enhanced by pyocyanine at 0.1 μg/ml and abolished by pyocyanine at 0.5 μg/ml (Fig. 6). Concentration-dependent effects of pyocyanine were also observed on SAC- and PWM-driven differentiation of the B cells to immunoglobulin-containing plasma cells. At low concentrations (0.05 and 0.1 μg/ml), up to threefold-larger numbers of plasma cells were found. Pyocyanine at 0.2 μg/ml was inhibitory, and 0.5 μg/ml abolished B-cell differentiation. Although the number of immunoglobulin-positive cells was enhanced after mitogenic stimulation in the presence of low concentrations of pyocyanine, this pigment inhibited the activation of immunoglobulin-secreting cells by SAC or PWM at all concentrations tested (Fig. 6). The experiments were run three times with cells from different donors. Figure 6 shows one of these experiments, giving typical results.

TABLE 1. Effect of pyocyanine on DNA synthesis and IL-2 receptor (CD25) expression by human T lymphocytes after stimulation with PHA[a]

PHA concn (μg/ml)	Pyocyanine concn (μg/ml)	DNA synthesis (cpm/culture)[b]	% of CD25-positive cells[c]	Fluorescence intensity (channel no.)[d]
0	0	60 ± 16	4[b]	246
1	0	5,045 ± 513	66	520
1	0.1	15,934 ± 1,631	68	507
1	0.5	168 ± 17	17	312
1	1.0	38 ± 5	6	302

[a] Human MNC were stimulated with PHA (1 μg/ml) in the presence or absence of pyocyanine. After a 4-day culture the DNA synthesis and expression of CD25 by the cells were determined.
[b] Mean ± standard deviation of triplicate cultures.
[c] Mean of triplicate cultures (standard deviation < 15%).
[d] Only the mean fluorescence intensities of CD25-positive cells are given. Results are means of triplicate cultures (standard deviation < 15%). Cells cultured in the presence of pyocyanine but in the absence of PHA showed no induction of CD25.

Effect of pyocyanine on LPS-induced monokine production by monocytes. Monokines such as IL-1 and TNF take part in specific and nonspecific inflammatory reactions of the host against bacteria. Therefore, it was of interest to investigate the effect of pyocyanine on the induction and release of these monokines by a bacterial cell wall compound. Human MNC were stimulated by LPS under serum-free conditions in the presence or absence of pyocyanine. Culture conditions used were always optimal for IL-1 and TNF production as judged in primary experiments. The culture supernatants were harvested after 24 h and tested for IL-1 and TNF activity in a bioassay. Figure 7 gives typical results of one of three experiments. We found that at all concentrations tested (0.1 to 1.0 μg/ml) pyocyanine enhanced the production of IL-1 after LPS stimulation of the cells (Fig. 7). Pyocyanine alone had only a slight effect on IL-1 production. LPS-induced TNF production by monocytes was not altered at 0.1 μg of pyocyanine per ml (Fig. 7), but at 0.2 μg/ml or higher, LPS-stimulated monocytes produced larger amounts of TNF. In contrast to IL-1 production, monocytes did not produce detectable amounts of TNF after stimulation with pyocyanine alone.

DISCUSSION

P. aeruginosa is a facultative pathogenic bacterium which causes serious hospital-acquired infections. Up to now, no single factor of the bacteria or host has been identified that is attributed comprehensively to the poor prognosis of the patients infected with this organism. Pyocyanine is one of the factors which may contribute to the virulence of *P. aeruginosa*. It has been shown that pyocyanine inhibits specific as well as nonspecific immune reactions in vitro (17, 20, 21, 30). This study was performed to investigate the influence of pyocyanine on the production of immunoglobulins by B lymphocytes, the secretion of IL-2 by T lymphocytes, and the secretion of IL-1 and TNF by monocytes. Furthermore, we measured the effect of pyocyanine on mitogen-induced proliferation of B and T lymphocytes and on CD25 expression by T lymphocytes.

In agreement with Sorensen et al. (30) and Mühlradt et al. (20), we found that the inhibitory effects of pyocyanine cannot be explained simply by a general toxicity. Below 0.5 μg/ml the pigment was not toxic during stimulation of MNC with PHA for 4 days by the trypan blue exclusion test.

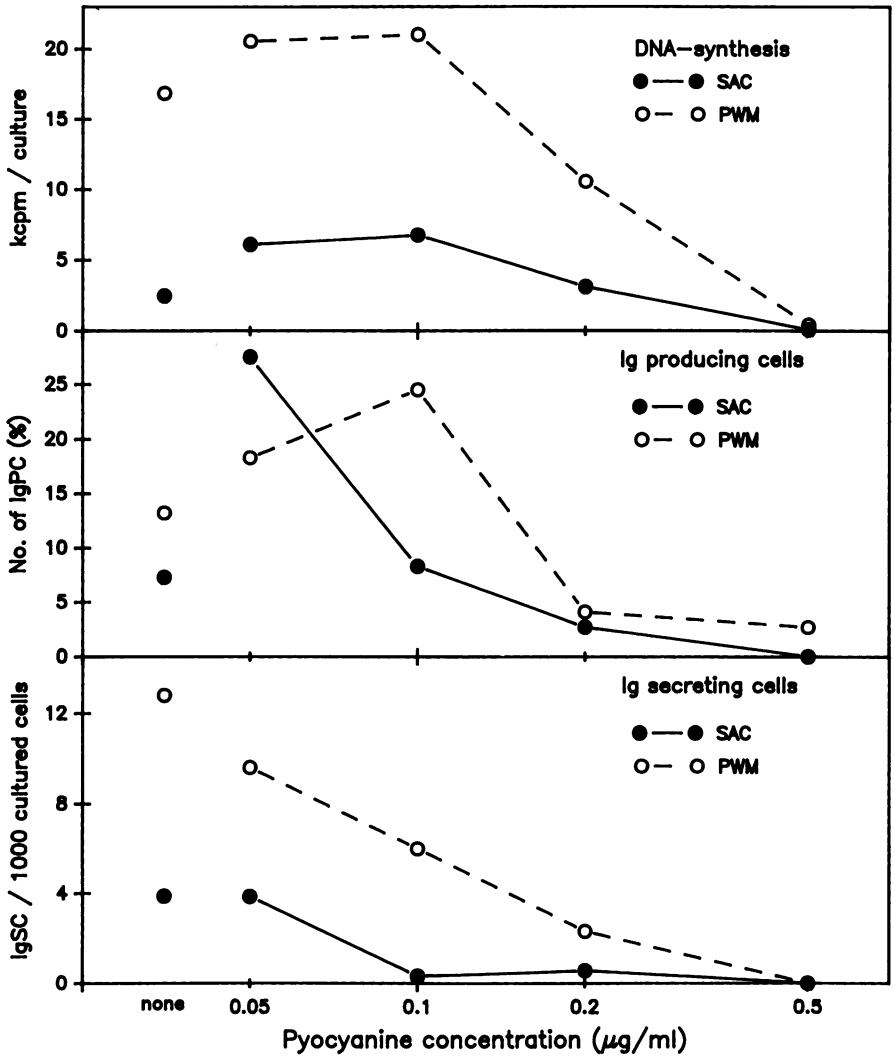

FIG. 6. Pyocyanine dose-response curves for mitogenic stimulation of B lymphocytes. Human MNC were stimulated by SAC (10 µg/ml) or PWM (0.4 µg/ml) in the absence or presence of various concentrations of pyocyanine. After a culture period of 3 days, DNA synthesis was measured. The number of IPC (IgPC) and of ISC (IgSC) was determined after 6 days of culture. The results are expressed as cpm per culture (DNA synthesis), as the percentage of IPC found in the harvested cells (number of IPC), or as the number of ISC per 1,000 seeded cells. Each value represents the mean of triplicate cultures. The standard deviation was less than 15%. When cells were cultured in the presence of various doses of pyocyanine in the absence of SAC or PWM, the values never exceeded the control values.

Furthermore, IL-2 production by T lymphocytes was not reduced but enhanced by the addition of up to 0.5 µg of pyocyanine per ml. However, pyocyanine was toxic when added at a high concentration (1 µg/ml). The total abrogation of DNA synthesis and IL-2 production at this high concentration of the phenazine was probably due to this toxicity. Whether pyocyanine may be toxic for a specific population of cells at lower concentrations cannot be excluded by the trypan blue exclusion assay.

We found that pyocyanine has stimulatory as well as inhibitory effects on the response of the cells to mitogens. Mitogen-induced proliferation of T and B lymphocytes was markedly enhanced by the addition of low concentrations (0.05 to 0.1 µg/ml) of the phenazine pigment. At this concentration the production of IL-2 by T cells was enhanced, whereas the expression of CD25 was not altered. We should, however, consider that the expression of CD25 does not have to correlate with the expression of the functional high-affinity heterodimeric IL-2 receptor. Since both the 55-kilodalton Tac antigen and the second (p75) chain of the IL-2 receptor are necessary for high-affinity binding of IL-2, we cannot assume an unaltered functional high-affinity IL-2-binding capacity.

Our results show that there is a dual dose-dependent inhibitory or stimulatory effect of pyocyanine on IL-2 production. The actual response of the cells (enhancement or suppression) depends on the concentration of pyocyanine used in the experiment. However, these findings are not able to resolve the discrepancy between the results of Mühlradt et al. (20) and those of Nutman et al. (21) on the basis of the concentration of pyocyanine used. Since Mühlradt et al. (20) found only stimulatory effects of pyocyanine on concanavalin A-stimulated IL-2 production even at a high concentration (2.63 µg of pyocyanine per ml), this fact may be simply explained by the use of murine spleen cells instead of human MNC (21), since their dose-response curves for pyocyanine may be different. We therefore postulate that murine T lymphocytes are less sensitive to the effect of pyocyanine

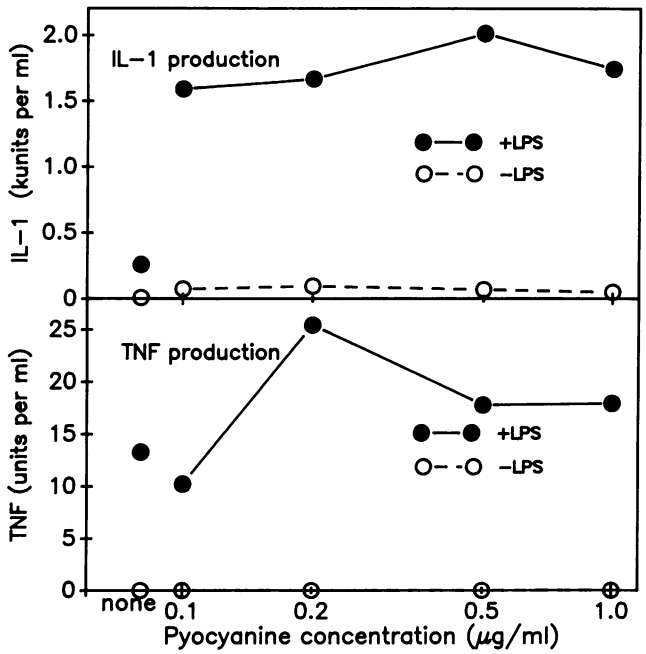

FIG. 7. Pyocyanine dose-response curves for monokine production by monocytes. Human MNC were stimulated by LPS (100 ng/ml) in the absence or presence of various amounts of pyocyanine. In further cultures LPS was absent but pyocyanine was present. After a 24-h culture, the supernatants were harvested and the concentrations of TNF and IL-1 were determined. The results are expressed as units per milliliter. Each value represents the mean of three cultures. The standard deviation was less than 15%.

leading to inhibition of IL-2 production than are human T lymphocytes.

IL-2 is necessary for the growth of T lymphocytes (29) and is also involved in the growth and differentiation of B lymphocytes (13). We therefore postulate that the higher concentration of this (and presumably other) growth factor(s) leads to an enhancement of the proliferative response of the cells. It seems unlikely that the high concentration of IL-2 found in the culture supernatants of T lymphocytes might simply be due to lower consumption of IL-2 by the responding cells, since the proliferative activity of the cells was even higher or at least not reduced by pyocyanine (up to 0.2 μg/ml). Stimulation of IL-2 production and of IL-2R expression is under the control of different signal pathways (11). We presume that pyocyanine may affect these different pathways in a different manner, leading to enhanced IL-2 production but to reduced IL-2R expression. Furthermore, pyocyanine enhances mitogen-induced IL-1 and TNF production (Fig. 7). It has been shown that both monokines act in concert with a mitogen to enhance markedly the transcription of genes for IL-2 and other lymphokines (9). Therefore, it is possible that enhanced IL-1 production by accessory monocytes-macrophages caused by pyocyanine leads to increased IL-2 gene transcription. Whether transcription or another activation pathway is directly or indirectly influenced by pyocyanine is not known.

Whereas the enhancement of T-lymphocyte response to PHA at 0.1 μg of pyocyanine per ml is accompanied by increased IL-2 production, the reduction of T-lymphocyte proliferation at higher concentrations seems to be caused by the inhibitory effect of the pigment on CD25 expression. The reduction of proliferation of T cells occurs in parallel with a diminished CD25 expression (Table 1). We therefore conclude that at this concentration of pyocyanine, signals or pathways leading to CD25 expression are affected. However, investigations of the effect of pyocyanine on the expression of the functional high-affinity IL-2 receptor consisting of the α-(p75) chain and the β-(p55) chain remain to be performed.

Pyocyanine added at different times during culture showed a dual effect not only when added at the beginning of the culture but also when added 24 h before the end of the culture (Fig. 3). This dual effect of pyocyanine on IL-2 production and DNA synthesis by PHA-stimulated T lymphocytes can also be observed in kinetics experiments (Fig. 4 and 5). This finding indicates that at least for T lymphocytes, pyocyanine did not simply shift the time course of the response but provided stimulatory or inhibitory signals during activation and response of the cells.

In contrast to the secretion of IL-2 by T lymphocytes, the secretion of immunoglobulins by B lymphocytes is reduced at low concentrations of pyocyanine (0.2 and 0.1 μg/ml, and in PWM-stimulated cultures even at 0.05 μg/ml). The inhibitory effect of low concentrations of pyocyanine on the B-cell response is directed solely against the secretion of immunoglobulins. Differentiation of the cells to plasma cells (IPC) and proliferation of the B lymphocytes after mitogenic stimulation are somewhat enhanced at low concentrations of pyocyanine. This indicates different steps leading to proliferation, differentiation, and secretion of immunoglobulins after mitogenic stimulation of B lymphocytes. Previously we claimed that the differentiation of B cells into IPC and ISC can be independently regulated by PWM-activated suppressor cells (25). It may therefore be that the differential response of B cells to mitogens in the presence of pyocyanine is due not only to effects of pyocyanine on B lymphocytes but also to a different sensitivity of interacting cells (for example, T helper or T suppressor cells).

As observed for T-lymphocyte responses, the response of B lymphocytes to SAC or to PWM can be inhibited by high doses of pyocyanine (0.2 μg/ml or higher). At these concentrations, not only immunoglobulin secretion but also intracellular immunoglobulin production and proliferation by the B cells are inhibited. These data again raise the question of whether pyocyanine acts at different steps of cellular activation. Considering that pyocyanine reduces IL-2R expression by T lymphocytes, we postulate that pyocyanine at a high concentration may reduce the expression of receptors for growth factors such as IL-2, IL-4, and/or IL-5, which are necessary for B-lymphocyte proliferation and differentiation. This presumption has yet to be verified.

Finally, we have investigated the effect of pyocyanine on the production of IL-1 and TNF by monocytes. These monokines are involved in unspecific inflammatory reactions of the host and mediate various fatal biological reactions, e.g., endotoxic shock (1, 4). Our experiments show that pyocyanine has no or only a marginal activity in inducing these monokines by itself, but it has profound effects on LPS-induced production of monokines. At 0.2 μg of pyocyanine per ml or higher, the production of IL-1 and TNF by LPS-stimulated monocytes was enhanced up to eight- and twofold, respectively. Even at 1.0 μg/ml, a concentration at which all T- and B-cell functions tested were abolished, pyocyanine was able to augment IL-1 and TNF secretion by LPS-stimulated monocytes. These findings indicate that pyocyanine is able to enhance inflammatory responses of the host, which may lead to irreversible shock.

All of our experiments on the effect of pyocyanine were

performed with unfractionated MNC. It remains to be clarified whether the observed influences of pyocyanine are indirect and mediated by other cells present in culture. Helper and/or suppressor T cells and monocytes have strong regulatory effects, and an effect of pyocyanine within this network may indirectly influence the response of T cells to PHA, of B cells to PWM or SAC, and/or of monocytes to LPS.

Our results and the results published by others (17, 20, 21, 30) demonstrate that pyocyanine inhibits reactions necessary for specific defense mechanisms of the host, such as proliferation of T lymphocytes, production of IL-2, expression of IL-2R, development of cytotoxic T lymphocytes, and secretion of antibodies. We therefore conclude that pyocyanine may contribute to the immunosuppressive action of *P. aeruginosa*. On the other hand, pyocyanine shows a concentration-dependent enhancement of IL-2 production by T lymphocytes (Fig. 2), of IL-1 and TNF production by monocytes-macrophages (Fig. 7), and of superoxide production by polymorphonuclear leukocytes (17). IL-1, TNF, IL-2, and superoxide production is involved in inflammatory reactions such as tissue destruction, septic shock, and production of acute-phase proteins (4, 5, 19). These dual pathophysiological reactions may lead to a fatal status of the infected host: the specific immune response leading to a killing of the bacteria may be inhibited by pyocyanine, thus prolonging the infection or preventing healing, whereas the enhanced production of mediators of inflammatory reactions might lead to harmful responses of the host (e.g., tissue damage, septic shock) rather than to a destruction of the bacteria. In this way pyocyanine might contribute, together with other toxins of *P. aeruginosa*, to the pathogenesis of infection with these bacteria. These rather hypothetical assumptions require further investigations to clarify the effect of pyocyanine on specific immune and unspecific inflammatory reactions.

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